

PCT

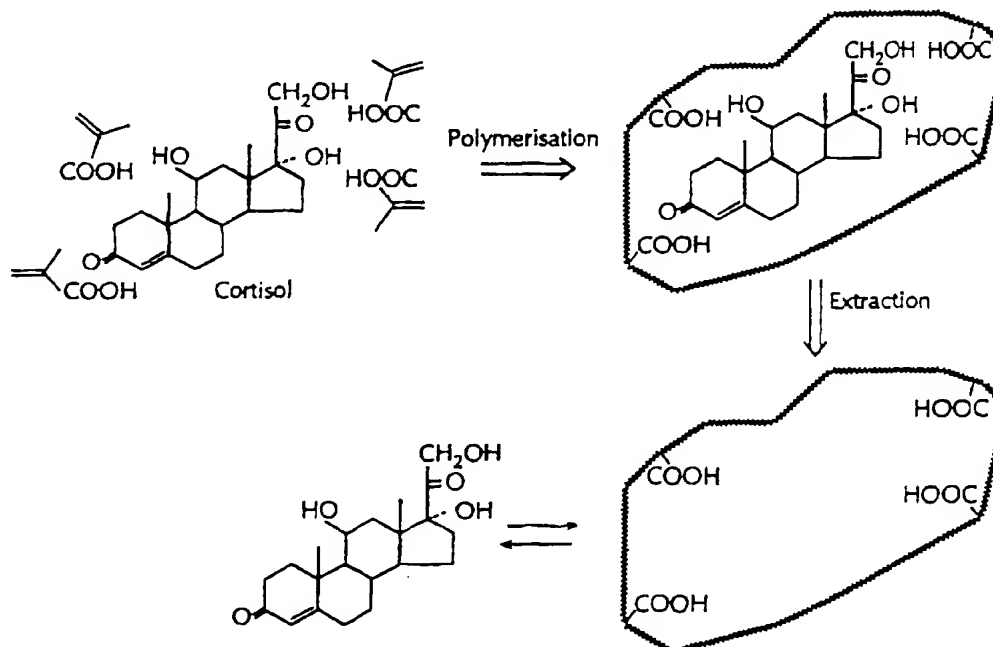
WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification ⁶ : C07K 16/44, 16/26, G01N 33/531, A61K 39/395, G01N 33/534, 33/53</p>	<p>A1</p>	<p>(11) International Publication Number: WO 97/38015 (43) International Publication Date: 16 October 1997 (16.10.97)</p>
<p>(21) International Application Number: PCT/US97/05151 (22) International Filing Date: 4 April 1997 (04.04.97) (30) Priority Data: 08/628,381 5 April 1996 (05.04.96) US (71) Applicant: IGEN, INC. [US/US]; 16020 Industrial Drive, Gaithersburg, MD 20877 (US). (71)(72) Applicants and Inventors: MOSBACH, Klaus [SE/SE]; Lackalanga 31, S-244 02 Furulund (SE). RAMSTRÖM, Olof [SE/SE]; Källarekroken 64, S-226 47 Lund (SE). (74) Agent: MARCUS, Michael, S.; Morgan & Finnegan, L.L.P., Suite 960 East, 1299 Pennsylvania Avenue, N.W., Washington, DC 20004 (US).</p>		<p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p>Published With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</p>

(54) Title: ARTIFICIAL ANTIBODIES TO CORTICOSTEROIDS PREPARED BY MOLECULAR IMPRINTING



(57) Abstract

The invention relates to artificial antibodies that are prepared by molecular imprinting, where methacrylic acid, ethylene glycol dimethacrylate and a print molecule are combined to form an artificial antibody having spatially positioned binding sites dictated by the corticosteroid print molecule, and the antibodies can be used in separation and analytical procedures.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon	KR	Republic of Korea	PL	Poland		
CN	China	KZ	Kazakhstan	PT	Portugal		
CU	Cuba	LC	Saint Lucia	RO	Romania		
CZ	Czech Republic	LI	Liechtenstein	RU	Russian Federation		
DE	Germany	LK	Sri Lanka	SD	Sudan		
DK	Denmark	LR	Liberia	SE	Sweden		
EE	Estonia			SG	Singapore		

**ARTIFICIAL ANTIBODIES TO CORTICOSTEROIDS
PREPARED BY MOLECULAR IMPRINTING**

FIELD OF THE INVENTION:

5 The present invention relates to artificial
antibodies that selectively recognize steroids and act as
antibody and receptor binding mimics. More specifically,
the present invention relates to molecularly imprinted
10 polymers (MIPs) that selectively recognize cortisol and
corticosterone based steroids, their preparation and use
in analyses, therapies and separation procedures.

BACKGROUND OF THE INVENTION:

15 Molecular imprinting is a technique devised to
generate a polymeric material that is analyte specific.
The analyte can be any organic molecule, biological or
macromolecule. Molecular imprinting has been used to
prepare materials that recognize proteins or other
20 biological compounds, especially where the structural
information needed for rational design is lacking.
Likewise, if a natural receptor is poorly characterized or
difficult to isolate, artificially prepared mimics may
serve as a useful alternative. Furthermore, such polymers
are considerably less costly to produce when compared to,
25 e.g., antibodies and receptors.

 Antibodies are used in several areas, such as
therapy, immunoaffinity and purification. Of particular
interest is the use of antibodies in immunoassays.
However, antibodies for these procedures are normally
30 produced by immunizing animals with the corresponding
antigen leading to polyclonal antibodies, or by using
fused cells (B cells) allowing the obtained cell lines to
produce monoclonal antibodies.

As an alternative, some non-biologically derived antibody mimics or artificial antibodies have been described. For example, the anti-theophylline and anti-diazepam polymers, i.e., mimics, prepared in accordance with the teachings of PCT Application WO 94/11403, the entirety of which is incorporated herein by reference. Such polymer structures are similar to biological antibodies in binding and recognizing antigens and avoid the need for animal sources. These antibody mimics are especially useful where it is difficult or impossible to raise antibodies.

The object of creating artificial counterparts to natural macromolecular binding entities, such as proteins, is of great interest. Employing natural macromolecules in rough environments such as high temperatures and pressures (e.g., sterilization conditions) is of major concern for many applications because of their natural sensitive properties. Furthermore, the efficiency and selectivity exerted by, e.g., receptors interacting with agonists and antagonists or antibodies recognizing antigens, is difficult to reproduce in synthetic systems [1]. Molecular imprinting provides an alternative to other approaches such as sophisticated procedures used in the field of supramolecular chemistry [2].

The rapidly mushrooming field of molecular imprinting is derived from the concept of creating designed recognition sites in macromolecular matrices by means of template polymerization [3-7]. Molecularly imprinted polymers have been shown to possess remarkable recognition properties that have been used in various fields such as drug separations [8-10], receptor mimics [11-14], bio-mimetic sensors [15], antibody mimics [16], template-assisted synthesis [17] and catalysis [18-19].

Of particular interest are the corticosteroids produced in the adrenal cortex and possess numerous and wide-spread effects in vivo. For example, the corticosteroids influence (1) metabolism, (2) electrolyte and water balance, (3) anti-inflammatory action, and (4) functions of the nervous system [20]. Many medical analyses where corticosteroids are of concern, e.g., in the assessment of the functional status of the adrenal cortex, utilize antibody-based assay methods such as RIA and ELISA for the selective recognition of a desired corticosteroid [21]. However, in addition to the general biological interest of steroid interactions with, e.g., antibodies and receptors, these substances are potentially useful for the study of molecular recognition phenomena [22]. The rigid structure of the fused ring system leads to a minimized number of conformations that the molecules may adopt in the interactions with recognition matrices resulting in higher binding strength since the entropy loss in binding is smaller [23] and a multitude of structurally very closely resembling structures are available. However, the limited number of polar interacting points necessary for non-covalent interactions inevitably leads to a decreased binding performance, and molecularly imprinted polymers against steroids have previously only been acquired using strong covalent binding systems such as carboxylic esters and carbonic acid esters [17,24].

Thus, a need exists for molecularly imprinted polymers (MIPs) that selectively recognizing steroid structures, steroid hormones, and in particular, steroids such as cortisol and corticosterone based steroids.

SUMMARY OF THE INVENTION:

The present invention relates to molecular imprinting as a tool for making polymers that mimic anti-corticosteroid antibody binding, the artificial antibodies, their preparation and use. Molecularly imprinted polymers were prepared against cortisol and corticosterone compounds and the ligand specificity was assayed using a radioimmunoassay technique. The binding characteristics of the cortisol and corticosterone imprinted polymers were estimated and equilibrium constants were determined.

An object of the present invention is to provide an artificial antibody, formed from polymerizable monomers, containing preset binding sites for a hormone steroid compound or derivative.

Another object of the present invention is to provide an artificial antibody, formed from polymerizable monomers, containing preset binding sites for a corticosteroid compound or derivative.

Another object of the present invention is to provide a process for preparing an artificial antibody, formed from polymerizable monomers, containing preset binding sites for a corticosteroid compound or derivative, i.e., print molecule.

A still further object is to provide a process where the polymerization of functional monomers is carried out in the presence of a porogenic solvent and, a corticosteroid print molecule which is non-covalently bound to the functional groups of the monomer and/or copolymer. Subsequent removal of the print molecule from the rigid polymer results in sites within the polymer that are complementary to and have an affinity for the original print molecule. The sites provide a preset or

predetermined spatial orientation of the polymer's functional groups to selectively separate a molecule or compound of interest.

5 A still further object of the present invention is to provide a process for separating steroids by using the antibody mimics according to the present invention.

Another object of the present invention is the use of the corticosteroid selective mimics in immuno and radioimmunoassay procedures.

10 A further object of the present invention is the use of artificial antibodies (corticosteroid selective mimics) in therapies and/or diagnoses, in which artificial antibodies are administered to a mammal body, which artificial antibodies consist of a biocompatible polymer
15 carrying specific binding sites mimicking the properties of antibodies towards an organic molecule.

These and other objects and advantages will become more apparent when considered with the following detailed description, non-limiting examples and drawings.

20 **BRIEF DESCRIPTION OF THE DRAWINGS:**

Fig. 1 shows a schematic of the molecular imprinting procedure according to the present invention.

25 Fig. 2 shows the chemical structure of the cortisol (hydrocortisone) print molecule.

Fig. 3 shows the chemical structures of the cortisone print molecule.

30 Fig. 4 shows the chemical structure of 21-deoxycortisol molecule.

35 Fig. 5 shows the chemical structure of corticosterone print molecule.

Fig. 6 shows the chemical structure of 11-deoxycortisol print molecule.

Fig. 7 shows the chemical structure of the prednisolone print molecule.

Fig. 8 shows the Scatchard plot for print species binding to imprinted polymers, i.e., cortisol binding by anti-cortisol polymer (MIP1), where B denotes the amount of bound ligand and F the amount of free ligand.

Fig. 9 shows the Scatchard plot for print species binding to imprinted polymers, i.e., corticosterone binding by anti-corticosterone binding by anti-corticosterone polymer (MIP3), where B denotes the amount of bound ligand and F the amount of free ligand.

Fig. 10 shows the dose-response curves for the print species interactions with molecularly imprinted polymers, i.e., cortisol binding by anti-cortisol polymer MIP1, where B denotes the amount of bound radiolabelled ligand and C is the concentration of the competing ligand.

Fig. 11 shows the dose-response curves for the print species interactions with molecularly imprinted polymers, i.e., corticosterone binding by anti-corticosterone polymer (MIP3), where B denotes amount bound radiolabelled ligand and C is the concentration of the competing ligand.

DETAILED DESCRIPTION OF THE INVENTION:

In the present invention molecularly imprinted polymers were prepared against cortisol and corticosterone compounds. The polymers are prepared from the copolymerization of a monomer that is negatively charged, such as methacrylic acid (MAA) $\text{CH}_2=\text{C}(\text{CH}_3)\text{COOH}$ or itaconic acid with cross-linking ethylene glycol dimethacrylate monomer, with azo-bisisobutyronitrile as the initiator and were obtained from Merck (Darmstadt, FRG). Other negatively charged monomers include, but are not limited to acrylic acid, maleic acid, vinylbenzoic acid and 2-trifluoromethyl acrylic acid. Although the preferred cross-linking monomer is ethylene glycol dimethacrylate, other cross-linking monomers may be used, such as, divinylbenzene and trimethylolpropane trimethacrylate (TRIM). Scintillation liquid, Ecoscint O, was from National Diagnostics (Manville, NJ, USA). All solvents were of either HPLC or analytical grade.

The cortisol and corticosterone compounds include, but are not limited to cortisol, deoxycortisol, 11-deoxycortisol, 21-deoxycortisol, corticosterone, 21-deoxycortisone, 11-dehydrocorticosterone, cortexolone, prednisolone, substituted prednisolone and cortisone. Cortisol, corticosterone, 21-deoxycortisone, cortexolone, prednisolone and cortisone were obtained from Sigma Chemical Co. (St. Louis, MO, USA). $[1,2,6,7\text{-}^3\text{H}]$ cortisol (specific activity 2.22 TBq/mmol) and $[1,2,6,7\text{-}^3\text{H}]$ corticosterone (specific activity 3.03 TBq/mmol) were from Amersham International plc. (Little Chalfont, UK). In addition to the corticosteroids identified above, print molecules based on other steroid hormones, such as the androgens, estrogens, progestins, and gonadotropin releasing hormones identified in Basic and Clinical Pharmacology, 4th Ed., 1989, pg. 696-700, incorporated herein by reference, may be used.

Ligand specificity was assayed using a radio-immunoassay technique and the binding characteristics of the cortisol and corticosterone imprinted polymers were estimated and equilibrium constants were determined.

5 The self-assembly imprinting protocol used in the present invention, where only non-covalent interactions are utilized in the formation and maintenance of the complexes between the functionally-active monomers and the print species, relies, to a large extent, upon the
10 solvent that is used. In the present invention, more polar solvents had to be used due to the low solubility of the print species in non-polar solvents that are conventionally used for increasing the selectivity of the
15 artificial recognition sites. Such conventional solvents include dichloromethane and toluene. The preferred polar solvents of the present invention include two different porogens, tetrahydrofuran and acetone. Other solvents may be used so long as they solubilize the steroid of interest and have the requisite polarity and could include
20 chloroform, ethylacetate, isopropanol and acetonitrile/

 The concentrations of the print species (molecules or compounds) in the protocols used was too high for reaching full solubility and were modified. Addition of functional monomer, i.e., methacrylic acid, to
25 adjust the ratio to preferably about 10:1 (functional monomer to print molecule) provided clear solutions. The clear solutions indicated the establishment of strong interactions between the functional monomer and the print molecule (species).

The molecularly imprinted polymers (MIPs) were prepared according to Table 1 below.

TABLE 1

Polymer	Print Molecule	Monomers (Ratio ^a)	Porogen
MIP1	cortisol	MAA/EDMA (10:50)	tetrahydrofuran
MIP2	cortisol	MAA/EDMA (10:50)	acetone
MIP3	cortico-sterone	MAA/EDMA (10:50)	tetrahydrofuran
REF1	none	MAA/EDMA	tetrahydrofuran
REF2	none	MAA/EDMA	acetone

^a Molar ratio relative to print molecule

The print molecule was dissolved in dry porogen, either tetrahydrofuran or acetone, together with the functional monomer, methacrylic acid. The cross-linking monomer, ethylene glycol dimethacrylate, and the initiating agent, azo-bisisobutyronitrile, were added and the solutions were chilled on an ice-bath and purged thoroughly with nitrogen for ten minutes. The degassed solution was photolytically polymerized under nitrogen atmosphere at 4°C overnight by use of a standard laboratory UV-source at 366 nm (CAMAG, Bubendorf, CH). The resulting polymer was crushed, ground in a mechanical mortar (Retsch, Haan, FRG) and wet-sieved (25:µm, Retsch) with water. Fine particles were removed through repeated sedimentation in acetone. The print species were extracted by extensive washing with a methanol/acetic acid solution (9:1, v/v), followed spectrophotometrically at 242 nm until no more print molecule could be detected.

As shown in Fig. 1, the print molecule (cortisol) is initially dissolved in the porogen (tetrahydrofuran or acetone) and allowed to form non-covalent complexes to the functional monomer (methacrylic

acid). Following addition of cross-linker (ethylene glycol dimethacrylate) and initiator (azo-bisisobutyronitrile), these complexes are arrested by polymerization. Finally, the print molecule is extracted by washing and the molecularly imprinted polymer is ready for association/dissociation studies. A procedure similar to that shown in Fig. 1 is followed for the compounds of Fig. 3 - cortisone; Fig. 4 - 21-deoxycortisol; Fig. 5 - corticosterone; Fig. 6 - 11-deoxycortisol; and Fig. 7 - prednisolone.

The capacity of the methacrylic acid/ethylene diglycol dimethacrylate (MAA/EGDA) polymers was measured by saturation studies. Radiolabelled ligand to an activity of 500 Bq was added to polymer particles ranging from a concentration of 0.03 to 20 mg/mL in a total volume of 1.0 mL solvent in polypropylene micro-centrifuge tubes (Brand, Wertheim, FRG). The binding was allowed to reach equilibrium at ambient temperature on a rocking table overnight. Subsequently, the polymer particles were removed from the samples by centrifugation at 10,000g for five minutes and 500 μ L of the supernatant was added to 10 mL of scintillation cocktail in 20 mL scintillation vials (National Diagnostics, Atlanta, GA, USA) and the radioactivity was measured using a model 2119 RACKBETA β -radiation counter (LKB Wallac, Solentuna, Sweden).

The competition assays were performed in a similar way. Non-radiolabelled (cold) ligand ranging from 0.01 to 250 μ g was mixed with 1.0 mg of polymer particles in polypropylene microcentrifuge tubes. Radiolabelled (hot) ligand to an activity of 500 Bq was added and the volume was made up to 1.0 mL with solvent. The samples were allowed to reach equilibrium overnight at ambient temperature on a rocking table. The amount of bound ligand was estimated after centrifugation at 10,000 g for five minutes and measuring the radioactivity of 500 μ L

supernatant by addition of the latter to 10 mL of scintillation liquid and measuring the radioactivity using a β -radiation counter. The concentration of ligand capable of displacing 50% of bound ligand (IC_{50}) was calculated using the computer software package EBDA/LIGAND (Elsevier-Biosoft, Amsterdam, NL).

The capacities of the molecularly imprinted polymers for the print species were investigated by saturation of the polymer with increasing amount of ligand. The assays were performed in several different solvents, but optimal binding performance were achieved with mixtures of tetrahydrofuran and n-heptane. In order to achieve a higher solubility of the ligands for further studies, a small amount of acetic acid was added to the solvent. The resulting imprinting performance, as measured by the saturation studies, revealed no difference between the imprinting porogens, tetrahydrofuran or acetone, but tetrahydrofuran was chosen as the best porogen because of a closer resemblance with the solvent used in the analysis system. The amount of polymer capable of binding 50% of added radiolabelled print species was similar in the imprinted polymers, ~1.4 mg in the anti-cortisol polymers (MIP1 and MIP2) and 2.0 mg in the anti-corticosterone polymer (MIP3). The corresponding values for the reference polymer (REF1) were 6.3 mg and 7.0 mg, respectively. Using a polymer concentration of 1 mg/mL, the bindings by the blank polymers were 10-16% of the binding by the imprinted polymers and this concentration level was chosen for further experiments.

The binding characteristics of the polymers are heterogeneous in nature, as reflected by the non-linear Scatchard plots shown in FIGS 8 and 9. This "polyclonal" behavior is an unavoidable effect from the imprinting procedure, in which the weak, non-covalent, interactions between the template molecules and the functional monomers

lead to the formation of gradually differing sites in the finished polymer. Thus, sites are formed of which the binding strength ranges from reasonably high affinity, exerted by a small amount of binding sites, down to larger number of sites with low affinity. Of the polymers studied, a two-site model can be readily employed to describe the binding of the templates to the polymers. The resulting figures of the dissociation constants and the corresponding binding site densities are displayed in Table 2 below.

TABLE 2

Polymer	K_D/M^{-1}		$B_{max}/(\mu\text{mol}\cdot\text{g}^{-1})$	
	High Affinity	Low Affinity	High Affinity	Low Affinity
MIP1	$0.18\cdot 10^{-6}$	$1.59\cdot 10^3$	0.21	280
MIP3	$1.23\cdot 10^{-6}$	$0.84\cdot 10^{-3}$	0.37	130

These values are of the same order for the polymers, where the two-site model is somewhat more pronounced in the anti-cortisol polymer as reflected by a smaller number of sites with higher affinity and a larger density of sites with a lower affinity than the anti-corticosterone polymer. In comparison to natural antibodies these binding constants are lower, possibly resulting from the experimental conditions used. If a more sensitive assay method could be used, it would be feasible to measure the stronger binding affinities of the best sites.

The selectivities of the artificial antibodies were estimated from the measures of 50% displacement of radiolabelled template species from the polymers by non-radiolabelled displacing ligand (IC_{50}). The experimental design is analogous to standard competitive immunoassays used, where unlabelled ligands compete with the radio-

labelled ligand for admission and binding to the sites. Dose-response curves obtained from experiments when unlabelled print species, cortisol or corticosterone, were used as competing ligands against MIP1 and MIP3 are displayed in FIGS. 10 and 11. The resulting values from the competition assays are presented in Table 3 together with the calculated cross-reactivities.

TABLE 3

Ligand	anti-cortisol polymer		anti-corticosterone polymer	
	IC ₅₀ /μM	Cross-reactivity	IC ₅₀ /μM	Cross-reactivity
Cortisol	0.27	100	2.2	10
Cortico-sterone	3.1	8.6	0.22	100
21-Deoxy-cortisol	6.7	4.0	41	0.54
11-Deoxy-cortisol	3.9	6.8	13	1.6
Predni-solone	0.74	36	3.8	5.7
Cortisone	30.0	0.89	57	0.38

From the structures of the various corticosteroids analyzed (FIGS. 2-7), the structural implications of the recognition can be deduced. For the anti-cortisol polymer (MIP1) the removal of one hydroxyl functionality of the imprinted structure leads to a decrease in binding as reflected by the increased IC₅₀-values. Removal of the 11-, 17-, or 21-OH groups, respectively, all reduce the binding by a factor of 11-25, where the 21-OH seems to be of higher importance for the recognition than the other hydroxyl groups. This can be viewed as the loss in

hydrogen bonding between the hydroxyl groups of the corticosteroids and the carboxyl groups of the surrounding polymer will inevitably reduce the binding.

5 By introduction of an additional unsaturation in the structure in the Δ -1 position, prednisolone, the binding is not as severely affected. The cross-reactivity is as high as 36% compared to cortisol. This can be understood as a minor locking of the flexibility of the A-ring will not drastically affect the binding to the sites.
10 A slight reduction in binding can be perceived since the prednisolone structure is unable to adapt to all of the configurations possible for cortisol.

On the other hand, changing the 11-OH group for a keto functionality as in cortisone, the binding is
15 unprecendently reduced. One reason for this effect can be changes in hydrogen bonding capabilities of the cortisone as compared to the cortisol molecule. In the cortisol structure the hydroxyl group is able to act as both hydrogen donor and hydrogen acceptor, thereby stabilizing
20 an interaction with the carboxyl functionality of the methacrylic acid residues of the polymer. The keto functionality of cortisone is unable to act as a hydrogen donor, leading to weaker interactions. Another explanation of this effect may be the sterical constraints of the
25 cortisone structure in comparison to cortisol. The planar keto functionality, as opposed to the hydroxyl pointing in the β -position, may lead to changes in ring structure.

For the anti-corticosterone polymer (MIP3),
30 introduction of an additional hydroxyl group in a 17- α -position (cortisol) reduces the binding to the polymer by a factor of 10. This is most probably due to steric constraints of the cortisol molecule to fit into the more tightly formed site of corticosterone. Although further changes from the basic corticosterone structure of the
35 other ligands studied, which differ from corticosterone in

two positions, lead to higher selectivity of the anti-corticosterone polymer in comparison to the anti-cortisol polymer, the tendencies of the structural implications for the recognition can be seen.

5 Similarly, as with the case of the anti-cortisol polymer, removal of one hydroxyl functionality from the template species, reduces the binding to the sites by a factor of 6-19. Also very similar to the anti-cortisol polymer is the reduction in binding resulting from the
10 introduction of a double bond in the A-ring (prednisolone), where the binding is reduced by 17%. The exchange of the 11- β -OH group for the planar keto group reduces binding by an additional 39%, much alike the binding by the anti-cortisol polymer.

15 In comparison to commercially used antibodies and antibodies reported in literature (Table 4), the artificial antibodies prepared by molecular imprinting exhibit strong similarities.

TABLE 4

Ligand	Cross-reactivity(%)					
	Cortisol Assays				Corticosterone Assays	
	ELISA ^a	RIA1 ^b	RIA2 ^c	RIA3 ^d	RIA4 ^e	RIA5 ^f
Cortisol	100	100	100	100	2.7	0.03
Corti- costerone	10	1.0	0.6	3.0	100	100
21-Deoxy- cortisol	<0.1	8.0	0.3	--	--	--
11-Deoxy- cortisol	19	--	--	--	--	--
Predni- solone	13	11	46	--	--	--
Cortisone	--	--	--	16	--	--

- ^a Monoclonal mouse anti-cortisol antibodies [27]
^b Sorin Biomedica, Almere, NL
^c Diagnostic Products Corporation, Los Angeles, CA
^d Polyclonal rabbit anti-cortisol serum [28]
^e Polyclonal rat anti-corticosterone serum [29]
^f ICN Biomedicals, Inc., Costa Mesa, CA

The cross-reactivities of the anti-cortisol antibodies are roughly in the same order as those obtained by the anti-cortisol polymer. Normally, the naturally-raised antibodies are either subjected to some sort of screening process, or monoclonal, thus leading to an optimized performance. In the case of molecularly imprinted polymers, a range of binding sites are obtained, i.e., the polymers can be perceived as "polyclonal" in appearance. This fact will inevitably reduce the binding selectivities.

5 The sensitivities of the assays using the artificial antibodies of the present invention, lie in the order of 10^{-7} - 10^{-8} M for cortisol and corticosterone using the anti-cortisol and anti-corticosterone polymers of the present invention, respectively, as indicated in the dose response curves displayed in FIGS 10 and 11. This limitation is in part a consequence of the detection method used and may be further forced by finding a more sensitive analysis.

10 Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious to those skilled in the art that certain changes and modifications may be practiced without
15 departing from the spirit and scope thereof as described in the specification and as defined in the appended claims.

REFERENCES:

- [1] H. Dugas, *Bioorganic Chemistry. A Chemical Approach To Enzyme Action*, 1989.
- [2] D.R. Smith, *Chem. Ind., Supramolecular Chemistry*, 1994, pp. 14-17.
- [3] K. Mosbach, et al., The Emerging Technique Of Molecular Imprinting And Its Future Impact On Biotechnology, *Bio/Technology*, 1996, 14, pp. 163-170.
- [4] R.J. Ansell, et al., Molecularly Imprinted Polymers For Bioanalysis: Chromatography, Binding Assays And Biomimetic Sensors, *Curr. Opin. Biotechnol.*, 1996, Vol. 7, pp. 89-94.
- [5] G. Wulff, Molecular Imprinting In Cross-Linked Materials With The Aid of Molecular Templates-A Way Towards Artificial Antibodies, *Angew. Chem. Int. Ed. Engl.*, 1995, Vol. 34, pp. 1812-32.
- [6] S. Vidyasankar, et al., Molecular Imprinting: Selective Materials For Separations, Sensors And Catalysis, *Curr. Opin. Biotechnol.*, Vol. 6, pp. 218-224, 1995.
- [7] K.J. Shea, Molecular Imprinting Of Synthetic Network Polymers: The De Novo Synthesis Of Macromolecular Binding And Catalytic Sites, *Trends In Polymer Science*, Vol. 2, pp. 166-173, 1994.
- [8] L. Fischer, et al., Direct Enantioseparation of β -Adrenergic Blockers Using A Chiral Stationary Phase Prepared By Molecular Imprinting, *J. Am. Chem. Soc.*, 1991, Vol. 113, pp. 9358-9360.
- [9] M. Kempe, et al., Direct Resolution Of Naproxen On A Non-Covalently Molecularly Imprinted Chiral Stationary Phase, *J. Chromatogr.*, 1994, Vol. 664, pp. 276-279.

- [10] K. Nilsson, et al., Imprinted Polymers As Antibody Mimics And New Affinity Gels For Selective Separations In Capillary Electrophoresis, *J. Chromatogr.*, 1994, Vol. 680, pp. 57-61.
- 5 [11] O. Ramstrom, et al., Synthetic Peptide Receptor Mimics: Highly Stereoselective Recognition In Non-Covalent Molecularly Imprinted Polymers, *Tetrahedron: Asymmetry*, 1994, Vol. 5, pp. 649-656.
- 10 [12] O. Ramstrom, et al., Chiral Recognition In Adrenergic Receptor Binding Mimics Prepared By Molecular Imprinting, *J. Mol. Recogn.*, 1995, In Press.
- [13] L.I. Andersson, et al., Mimics Of The Binding Sites Of Opioid Receptors Obtained By Molecular Imprinting Of Enkephalin And Morphine, *Proc. Natl. Acad. Sci.*, 15 1995, Vol. 92, pp. 4788-4792.
- [14] L.I. Andersson, Development Of Aqueous Buffer And Organic Solvent Based Radioligand Binding Assays For (S)-Propranolol, *Anal. Chem.*, 1996, Vol. 68, pp. 111-117.
- 20 [15] Kriz, et al., Introducing Biomimetic Sensors Based On Molecularly Imprinted Polymers As Recognition Elements, *Anal. Chem.*, 1995, Vol. 67, pp. 2142-2144.
- [16] G. Vlatakis, et al., Drug Assay Using Antibody Mimics Made By Molecular Imprinting, *Nature*, 1993, Vol. 361, 25 pp. 645-647.
- [17] S.E. Bystrom, et al., Selective Reduction Of Steroid 3- And 17-Ketones Using LiAlH₄ Activated Template Polymers, *J. Am. Chem. Soc.*, 1993, Vol. 115, pp. 2081-2083.
- 30 [18] R. Muller, et al., Molecularly Imprinted Polymers Facilitating A β -Elimination Reaction, *Makromol. Chem., Rapid Commun.*, 1993, Vol. 14, pp. 637-641.

- 19] J.V. Beach, et al., Designed Catalysts. A Synthetic Network Polymer That Catalyzes The Dehydrofluorination Of 4-Fluoro-4-(p-nitrophenyl)butan-2-one, J. Am. Chem. Soc., 1994, Vol. 116, pp. 379-380.
- 5 [20] L.S. Goodman, et al., The Pharmacological Basis Of Therapeutics, Pergamon Press, Inc., 1990, 8th Ed.
- [21] L.A. Kaplan, et al., Clinical Chemistry: Theory, Analysis And Correlation, C.V. Mosby Co., 1984.
- 10 [22] I.A. Nicholls, Thermodynamic Considerations For The Design Of And Ligand Recognition By Molecularly Imprinted Polymers, Chem. Lett., 1995, pp. 1035-1036.
- [23] W.P. Jencks, On The Attribution And Additivity Of Binding Energies, Proc. Natl. Acad. Sci. USA, 1981, Vol. 78, pp. 4046-4050.
- 15 [24] M. Whitcombe, et al., A New Method For The Introduction Of Recognition Site Functionality Into Polymers Prepared By Molecular Imprinting: Synthesis And Characterization Of Polymeric Receptors For Cholesterol, J. Am. Chem. Soc., 1995, Vol. 117, pp. 7105-7111.
- 20 [25] K. Mosbach, et al., Preparation And Application Of Polymer-Entrapped Enzymes And Microorganisms In Microbial Transformation Process With Special Reference To Steroid 11- β -Hydroxylation And Δ^1 -Dehydrogenation, Biotech. Bioeng., 1970, Vol. 12, pp. 19-27
- 25 [26] S.B. Mahato, et al., Steroid Transformations By Microorganisms III, Phytochemistry, 1989, Vol. 1, pp. 7-40.
- 30 [27] Lewis, J.G., Manley, L., Whitlow, J.C. & Elder, P.A. 1992. Production of a monoclonal antibody to cortisol: application to a direct enzyme-linked immunosorbent assay of plasma. Steroids 57, 82-85.
- 35

- [28] Purchas, R.W., Zinn, S.A. & Tucker, H.A. 1985. A simple method for separating unbound and bound cortisol in a radioimmunoassay. *Anal.Biochem.* 149, 399-403.
- 5 [29] Sainio, E-L., Lehtola, T. & Roininen, P. (1988). Radioimmunoassay of total and free corticosterone in rat plasma: measurement of the effect of different doses of corticosterone. *Steroids* 51, 609-622.

WE CLAIM:

1. Artificial antibodies comprising copolymers having preset-positioned selectively, specific binding sites for steroids.
2. The artificial antibodies according to claim 1, wherein said steroids are steroid hormones.
3. The artificial antibodies according to claim 1, wherein said steroids are corticosteroids.
4. The artificial antibodies according to claim 1, wherein said copolymers are prepared by copolymerizing a monomer carrying functional groups with a crosslinking monomer in the presence of a porogenic solvent.
5. The artificial antibodies according to claim 1, wherein said functional group monomer is a carboxylic acid.
6. The artificial antibodies according to claim 5, wherein said carboxylic acid is selected from methacrylic acid or itaconic acid.
7. The artificial antibodies according to claim 5, wherein said crosslinking monomer is ethylene glycol dimethacrylate.

8. The artificial antibodies according to claim 3, wherein the sites correspond to binding sites generated by a print molecule selected from cortisol, deoxycortisol, 11-deoxycortisol, 21-deoxycortisol, corticosterone, 21-deoxycortisone, 11-dehydrocorticosterone, cortexolone, prednisolone, substituted prednisolone or cortisone.

9. A copolymer comprising an artificial anti-corticosteroid antibody formed from methacrylic acid and ethylene glycol dimethacrylate and having pre-determined, spatially positioned specific binding sites for a steroid.

10. The copolymer according to claim 9, wherein said steroid is cortisol, deoxycortisol, 11-deoxycortisol, 21-deoxycortisol, corticosterone, 21-deoxycortisone, 11-dehydrocorticosterone, cortexolone, prednisolone, a substituted prednisolone or cortisone.

11. The artificial antibodies according to claim 9, wherein the polymers are biocompatible.

12. A method for producing an artificial polymeric antibody comprising:

i) combining a first monomer carrying functional groups with a steroid print molecule in the presence of a porogenic solvent;

ii) adding a crosslinking monomer to the monomer and print molecules of said step i);

iii) polymerizing said monomers to form a polymeric antibody; and

iv) removing the print molecules after step iii) to form an artificial polymeric antibody having spatially positioned, specific binding sites for steroids.

13. A method according to claim 12, characterized in that the print molecule is non-covalently bound to the copolymer.

5 14. A method according to claim 13, wherein said functional group containing monomer is selected from the group consisting of negatively charged monomers.

10 15. A method according to claim 12, wherein the crosslinking monomer is ethylene glycol dimethacrylate.

15 16. A method according to claim 12, wherein said functional group containing monomer is methacrylic acid.

17. A method according to claim 12, wherein said porogenic solvent is tetrahydrofuran or acetone.

20 18. A method according to claim 17, wherein said porogenic solvent is tetrahydrofuran.

25 19. A method for determination of an organic or biological molecule in a fluid sample, characterized in that a known amount of the organic molecule provided with a label is added to the sample, the sample is contacted with artificial antibodies according to claim 1.

30 20. An analysis method for steroid molecules comprising contacting a fluid suspected of containing a steroid with the artificial antibodies of claim 1.

35 21. The method according to claim 20, wherein said analysis in an immunoassay procedure.

22. The method according to claim 20, wherein said analysis in a radioimmunoassay procedure.

5 23. The method according to claim 20, wherein said analysis is a competitive binding assay.

24. The method according to claim 20, wherein said steroid is a steroid hormones.

10 25. The method according to claim 20, wherein said steroid is a corticosteroid.

15 26. A method of therapy or diagnosis, comprising administration of artificial antibodies to a mammal body, which artificial antibodies comprise a biocompatible polymer carrying specific binding sites mimicking the properties of antibodies towards a steroid.

20 27. The method according to claim 26, wherein said steroid is a steroid hormone.

28. The method according to claim 26, wherein said steroid is a corticosteroid.

25 29. The method according to claim 12, wherein said steroid is a steroid hormone.

30 30. The method according to claim 12, wherein said steroid is a corticosteroid.

35 31. A method of separating comprising contacting a fluid with a polymer carrying specific binding sites mimicking the properties of antibodies towards a steroid hormone, and isolating said steroid of interest.

32. A method of separating comprising contacting a fluid with a polymer carrying specific binding sites mimicking the properties of antibodies towards a corticosteroid, and isolating said corticosteroid of interest.

33. Artificial antibodies comprising copolymers having preset-positioned selectively, specific binding sites for a steroid hormone, wherein said copolymers are prepared by copolymerizing a monomer carrying functional groups with a crosslinking monomer in the presence of a porogenic solvent.

34. Artificial antibodies comprising copolymers having preset-positioned selectively, specific binding sites for a corticosteroid, wherein said copolymers are prepared by copolymerizing a monomer carrying functional groups with a crosslinking monomer in the presence of a porogenic solvent.

35. The artificial antibody of claim 33, wherein said steroid hormone is an androgen, an estrogen, or a progestin.

1/4

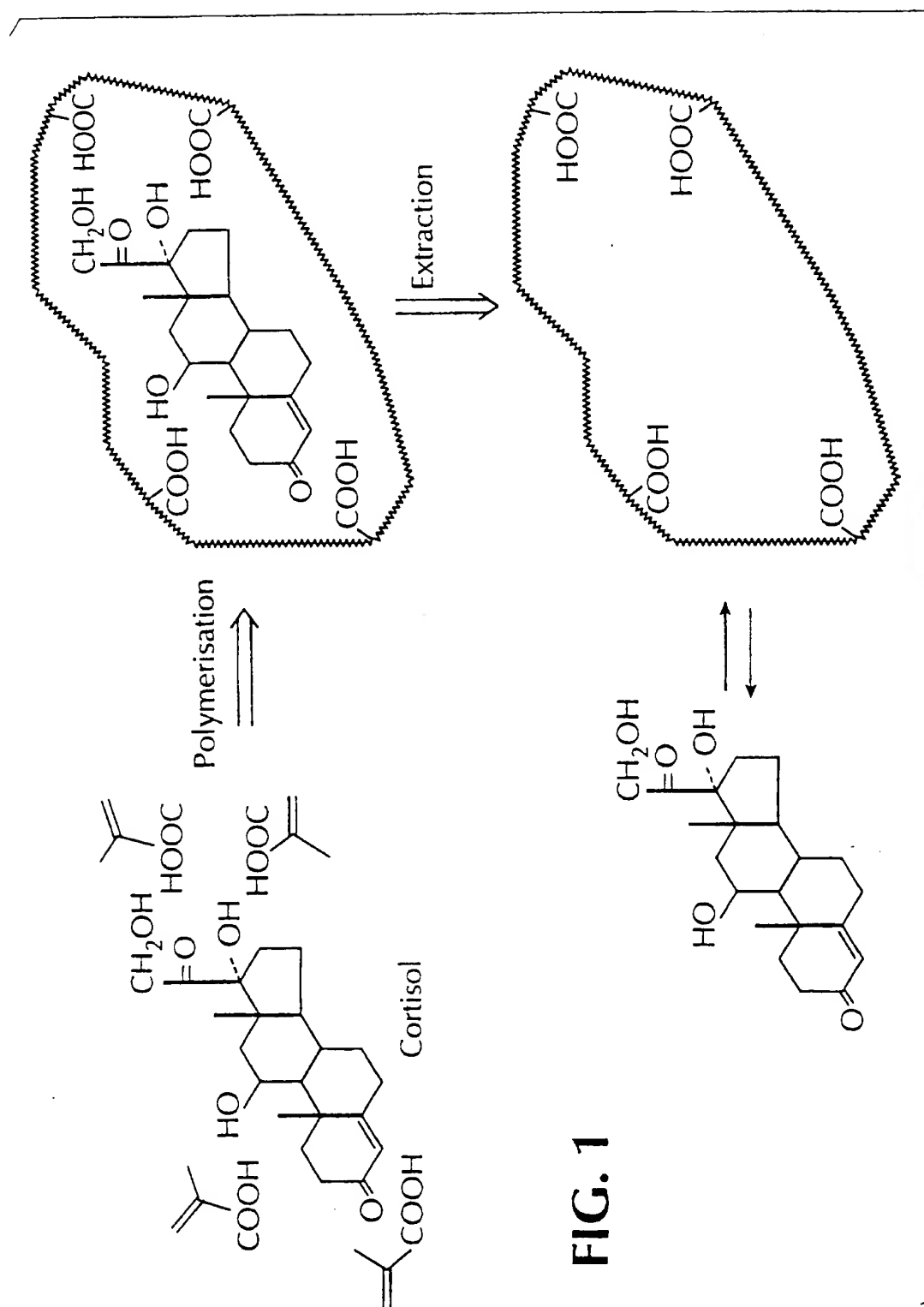
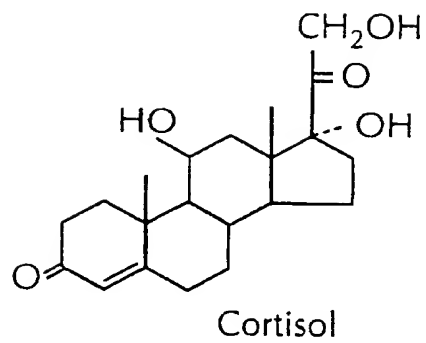
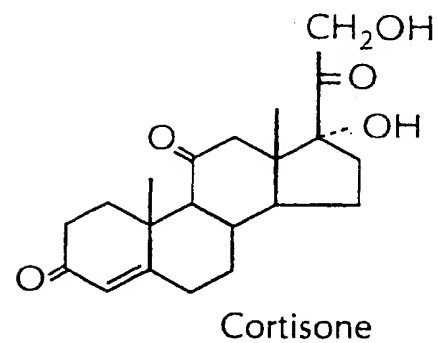
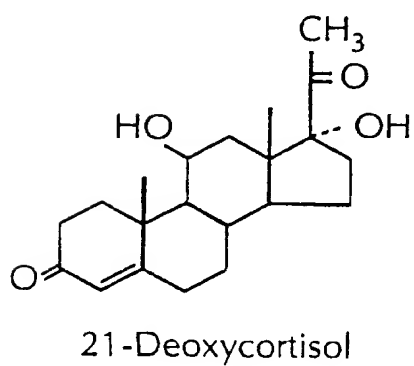
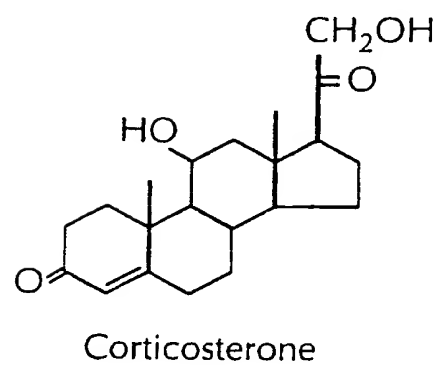
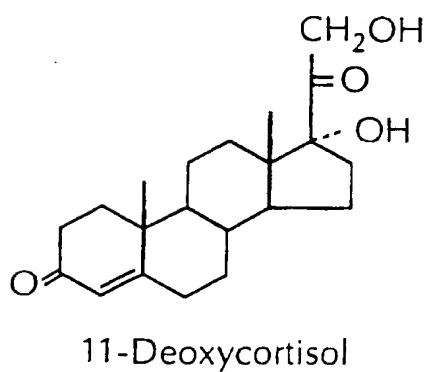
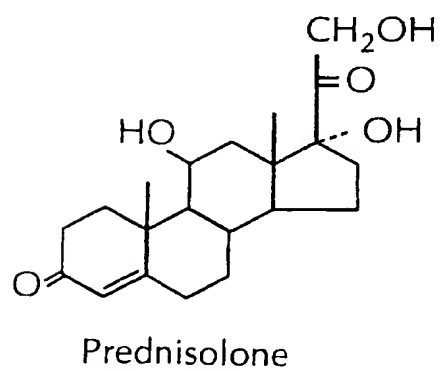


FIG. 2**FIG. 3****FIG. 4****FIG. 5****FIG. 6****FIG. 7**

3/4

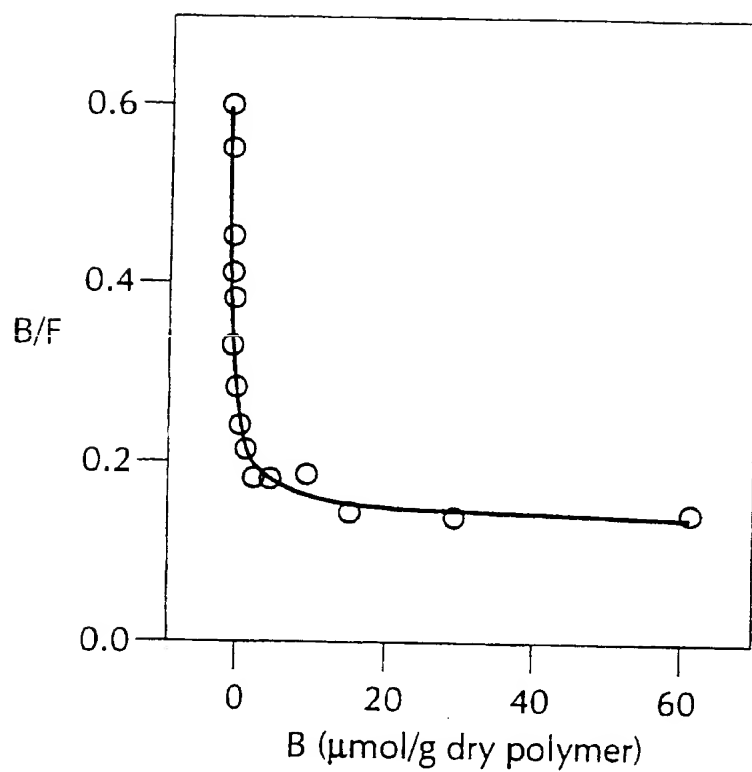
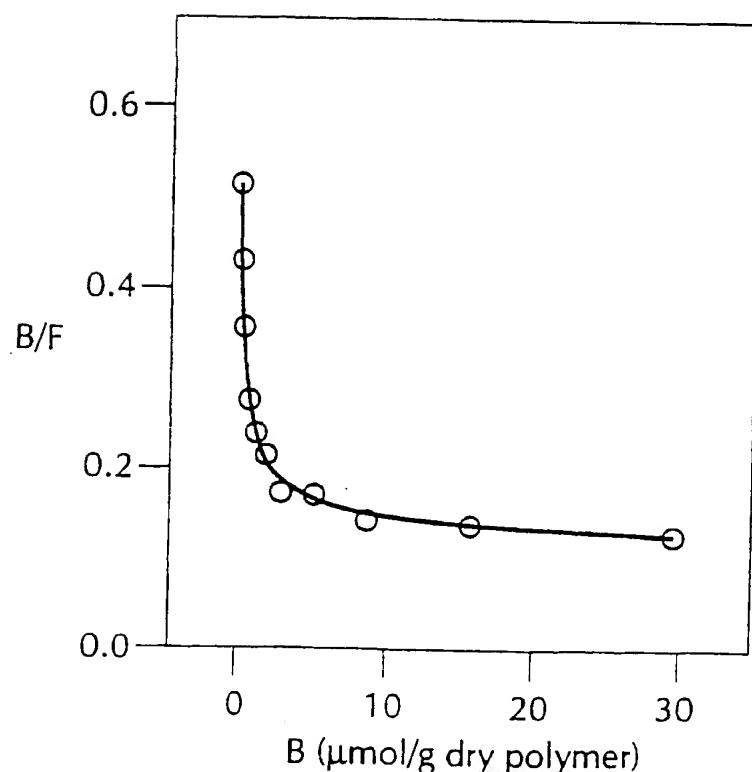
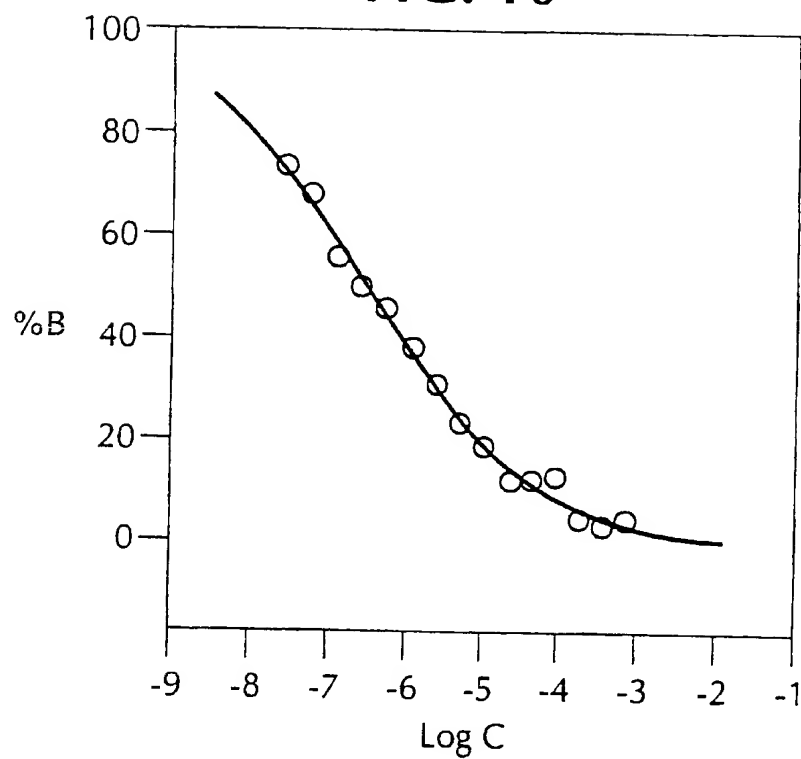
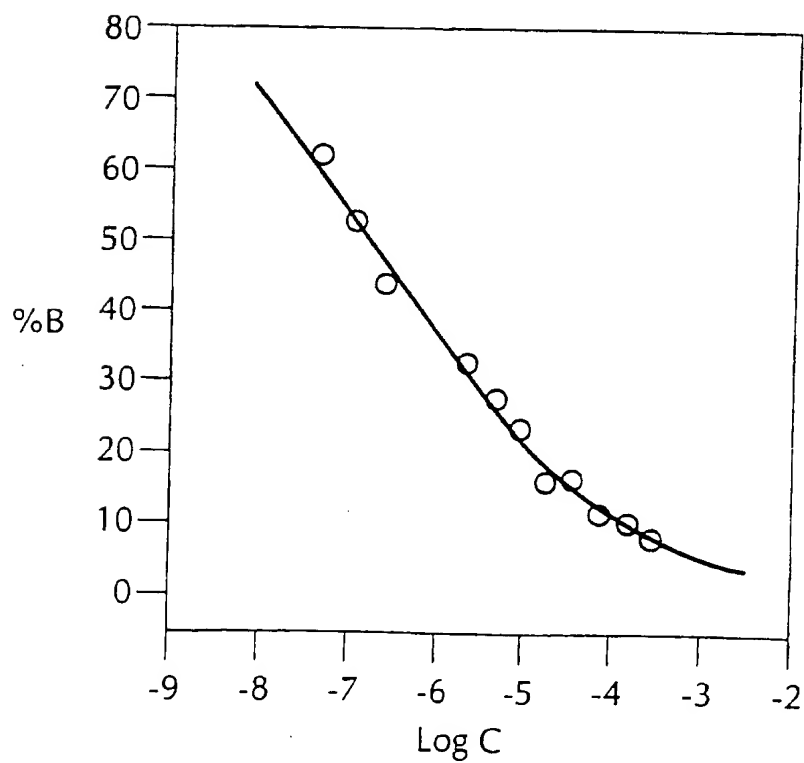
FIG. 8**FIG. 9**

FIG. 10**FIG. 11**

INTERNATIONAL SEARCH REPORT

Intern: al Application No. PCT/US 97/05151

A. CLASSIFICATION OF SUBJECT MATTER C 07 K 16/44, C 07 K 16/26, G 01 N 33/531, A 61 K 39/395, G 01 N 33/534, G 01 N 33/53		
According to International Patent Classification (IPC) or to both national classification and IPC 6		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) C 07 K, G 01 N, A 61 K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	CHEMICAL ABSTRACTS, vol. 125, no. 11, issued 1996, September 09, (Columbus, Ohio, USA), O. RAMSTROEM et al. "Arti- ficial antibodies to corticosteroids prepared by molecular imprinting" page 189, column 1, no. 133 002y; & Chem. Biol. 1996, 3(6), 471-477 (Eng). --	1-25, 29-30, 33-35
A	WO, A, 94/11 403 (MOSBACH) 26 May 1994 (26.05.94), claims 1-23. --	1-35
A, P	CHEMICAL ABSTRACTS, vol. 124, --	1-25,

☒ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *&* document member of the same patent family

Date of the actual completion of the international search
07 August 1997

Date of mailing of the international search report
28 -08- 1997

Name and mailing address of the ISA
European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+ 31-70) 340-2040, Tlx. 31 651 epo.nl,
Fax (+ 31-70) 340-3016

Authorized officer
SCHNASS e.h.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 97/05151

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	<p>no. 24, issued 1996, June 10, (Columbus, Ohio, USA), M.T. MULDOON et al. "Plastic antibodies: molecularly- -imprinted polymers" page 75, column 2, no. 318 916x; & Chem. Ind. (London) 1996, (6), 204-7 (Eng). -----</p>	<p>29, 30, 33-35</p>

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 97/05151

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: Remark:
because they relate to subject matter not required to be searched by this Authority, namely:
Though the subject-matters of the claims 26-28 are excluded from patentability by rule 39.1(iv)PCT as they concern methods for treatment of the human or animal body by therapy and in vivo diagnosis, these claims were searched, too.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

ANHANG

zum internationalen Recherchen-
bericht über die internationale
Patentanmeldung Nr.

ANNEX

to the International Search
Report to the International Patent
Application No.

ANNEXE

au rapport de recherche inter-
national relatif à la demande de brevet
international n°

PCT/US 97/05151 SAE 159428

In diesem Anhang sind die Mitglieder
der Patentfamilien der in obenge-
nannten internationalen Recherchenbericht
angeführten Patentdokumente angegeben.
Diese Angaben dienen nur zur Unter-
richtung und erfolgen ohne Gewähr.

This Annex lists the patent family
members relating to the patent documents
cited in the above-mentioned inter-
national search report. The Office is
in no way liable for these particulars
which are given merely for the purpose
of information.

La présente annexe indique les
membres de la famille de brevets
relatifs aux documents de brevets cités
dans le rapport de recherche inter-
national visée ci-dessus. Les renseigne-
ments fournis sont donnés à titre indica-
tif et n'engagent pas la responsabilité
de l'Office.

In Recherchenbericht angeführtes Patentdokument Patent document cited in search report Document de brevet cité dans le rapport de recherche	Datum der Veröffentlichung Publication date Date de publication	Mitglied(er) der Patentfamilie Patent family member(s) Membre(s) de la famille de brevets	Datum der Veröffentlichung Publication date Date de publication
WD A1 9411400	26-05-94	AU A1 54397/94	08-06-94
		CA A1 2149043	26-05-94
		EP A1 6699403	06-06-95
		FR A1 8508320	09-07-96
		IT AC 9203435	11-11-92